

Beneficial n-3 PUFA Levels and n-6:n-3 ratios after n-3 supplementation associated with  
reduced CRP: A pilot study in young adults

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## **I. Statement of the Problem**

### **Introduction**

Epidemiological studies show that current Western diets contain substantially greater amounts of n-6 polyunsaturated fatty acids (PUFA) relative to n-3 PUFAs (A. P. Simopoulos, 2000; A. P. Simopoulos, 2002a; A. P. Simopoulos, 2008). At present, the estimated n-6:n-3 ratio in the U.S. is 15:1 to 20:1, which differs dramatically from the 1:1 to 1:2 ratios of our ancestors (A. P. Simopoulos, 1999; A. P. Simopoulos, 2008). This change has evolved along with the significant modifications to the U.S. food supply that have occurred over the last 100-150 years. The rising n-6:n-3 ratio is a public health concern because PUFA metabolites modulate inflammation and, in general, n-6 PUFA metabolites are associated with stronger proinflammatory and immunoactive effects than those from n-3 PUFAs (Calder, 2010). Moreover, because an excessive, prolonged inflammatory response has been associated with the development and exacerbation of numerous chronic diseases (Fetterman & Zdanowicz, 2009), a more balanced dietary intake of n-6 and n-3 PUFAs is desired for optimal health. Evaluating the n-6:n-3 ratios in young adults is a beginning step in determining if strategies that promote a balanced ratio could help prevent the development of the many chronic inflammatory diseases in later years.

### **Background**

Consuming higher quantities of n-3 PUFAs is one approach for normalizing high n-6:n-3 ratios. Importantly, there is mounting evidence that increasing dietary n-3 PUFAs, particularly the long-chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is effective in treating and lowering the risk of developing inflammatory-related conditions (Fetterman & Zdanowicz, 2009), such as arthritis (Hurst, Rees, Randerson, Caterson, & Harwood, 2009),

cardiovascular heart disease (CVD) (Nair & Connolly, 2008), inflammatory bowel disease (Uchiyama et al., 2010), asthma (Schubert et al., 2009), and even sepsis (Barbosa, Miles, Calhau, Lafuente, & Calder, 2010). The anti-inflammatory actions of EPA and DHA are associated with their ability to (1) modulate the production and activity of inflammatory mediators such as eicosanoids from n-6 and n-3 metabolism; (2) alter cell membrane structure and function; (3) modulate molecules such as cytokines that are involved in normal and pathological cell functions; and (4) directly suppress the expression of genes involved in inflammation such as interleukin (IL)-1 and tumor necrosis factor (TNF)- $\alpha$  (Deckelbaum, Worgall, & Seo, 2006; A. P. Simopoulos, 1996). N-3 EPA is the preferred substrate when compared to n-6 AA (Figure 1) in the metabolic pathway and thus a diet rich in n-3 EPA is believed to shift the physiological state to one that is less inflammatory than that of a diet containing high amounts of n-6 AA (Harris, Hill, & Kris-Etherton, 2010).

### **Purpose**

The purpose of this study was to test the hypotheses that in healthy younger adults, (1) higher plasma concentrations of n-3 PUFAs and higher ratios of n-6:n-3 PUFAs are associated with greater systemic inflammation, as measured by C-reactive protein (CRP) concentrations, and (2) that oral supplementation with EPA and DHA is associated with rising plasma concentrations of n-3 PUFAs and decreasing n-6:n-3 ratios. These data are important to increasing our understanding of PUFAs' modulating effects on chronic diseases characterized by a proinflammatory status and how adding certain PUFAs to the diets of healthy young adults may lower their risk of developing these conditions.

**Significance**

Although excessive, prolonged inflammation is known to play an important role in the pathology of many diseases such as arthritis, the role of inflammation in CVD has only recently begun to be understood. Research suggests that systemic inflammation may be just as important to the development of heart disease as elevated levels of serum cholesterol (Taubes, 2002) because inflammation promotes all stages of atherosclerosis (Clearfield, 2005). The anti-inflammatory actions of EPA and DHA explain in part study findings indicating that an increased consumption of n-3 PUFAs is associated with lower cardiovascular morbidity and mortality independent of other known CVD risk factors (Breslow, 2006; Bucher, Hengstler, Schindler, & Meier, 2002) and a statistically significant reduction in cardiovascular risks (Breslow, 2006; Nair & Connolly, 2008). Furthermore, increasing EPA and DHA intake has been shown to reduce plasma triglycerides (Cleland, Caughey, James, & Proudman, 2006) and arterial stiffness (Kris-Etherton, Harris, & Appel, 2003), increase heart rate variability (Kris-Etherton et al., 2003), decrease the risk of dysrhythmias (Kris-Etherton et al., 2003; Lichtenstein et al., 2006) and reduce the synthesis of prothrombotic platelet thromboxanes and pro-inflammatory prostaglandins in whole blood (Cleland et al., 2006).

Because unremitting systemic inflammation is a contributing factor to many chronic diseases like CVD, quantifying systemic inflammation can aide in risk assessment and in determining the effectiveness of interventions designed to reduce inflammation. C-reactive protein (CRP) is a proinflammatory molecule produced by the liver that is often used to gauge the level of systemic inflammation. In conditions of chronic inflammation, CRP levels remain elevated (Taubes, 2002). Interestingly, studies have reported an association between lower CRP levels and an increased dietary intake of EPA and DHA (Breslow, 2006; Fetterman &

Zdanowicz, 2009; Martinez & Gonzalez-Juanatey, 2009). However, dietary intake studies of PUFAs should be balanced with studies that evaluate the association between physiological PUFA levels and inflammatory markers like CRP because this connection has not been definitively established and because some studies have reported only a modest to good correlation between dietary intake of PUFAs and PUFA blood levels (Fusconi et al., 2003; Seierstad et al., 2005).

### **Research Questions**

Our study was designed to answer the following research questions in a young adult sample: (1) Are higher plasma concentrations of n-3 PUFAs and higher ratios of n-6:n-3 PUFAs associated with greater systemic inflammation, as evidenced by increased CRP concentration? (2) Is oral n-3 EPA and DHA supplementation correlated with rising n-3 PUFA concentrations and decreasing n-6:n-3 ratios over time? And (3) Is EPA and DHA supplementation associated with decreasing CRP levels over time?

## **II. Review of Literature**

There is an increasing number of studies linking excessive, prolonged inflammation with the development of several of the chronic diseases that are prevalent in Western culture, such as heart disease, diabetes, asthma, and arthritis (Fetterman & Zdanowicz, 2009; A. P. Simopoulos, 2002b; A. P. Simopoulos, 2008; Taubes, 2002). Studying the younger adult sector to determine methods to decrease inflammation, such as supplementing diets with n-3 PUFAs in an attempt to balance high n-6:n-3 ratios, may help prevent many of these inflammatory-related diseases.

Cardiovascular disease is responsible for one in three (approximately 800,000) deaths reported each year in the U.S., with overall healthcare-related cost estimated at \$444 billion

dollars (Center for Disease Control (CDC), 2011). There is a growing body of evidence showing that systemic inflammation may be just as important to the development of heart disease as high levels of serum cholesterol (Taubes, 2002). An overly aggressive response to inflammation causes cells in the blood vessel walls to initiate the first stages of atherosclerosis. In cardiovascular disease, excess low-density lipoprotein (LDL) particles from the blood settle in blood vessel walls. The body's immune system identifies these particles as foreign, initiating an inflammatory response. Together, the LDL particles and inflammatory cells settle as plaques in the blood vessel wall. As inflammatory chemicals weaken the plaques over time, the body initiates clotting to control the rupture of the plaques. Clots then build up in the vessel, affecting blood flow (Underwood, 2005). According to the Centers for Disease Control and Prevention and the American Heart Association, multiple steps in atherogenesis are associated with an increased inflammatory response (Pearson et al., 2003).

### **Inflammation and Diabetes**

According to the American Diabetes Association, roughly 18.8 million people in the United States have diabetes, contributing to an annual cost of more than \$174 billion to the healthcare system (American Diabetes Association, 2011a). Inflammation is a primary component in the development of diabetes. Several of the major risk factors for Type 2 Diabetes such as obesity, a sedentary lifestyle, sleep deprivation and depression have been shown to contribute to local or systemic inflammation (Kolb & Mandrup-Poulsen, 2010). For example, in people who are overweight, fat cells grow more biochemically active, leading to an increase in the generation of inflammatory compounds such as TNF-alpha, IL-1 and resistin. When obesity contributes to inflammation, this inflammation promotes a key feature of diabetes—insulin resistance. Exactly how inflammation leads to insulin resistance is still unclear (Underwood,

2005), but a recent study funded by the American Diabetes Association reported that inflammation does indeed play a major role (American Diabetes Association, 2011b). This study suggests that the increased systemic inflammation found in obese individuals is linked to the activity of certain immune cells found in fat tissue. These immune cells can also be linked to insulin resistance. With insulin resistance, the body's cells do not respond to insulin, thus causing higher levels in insulin and high blood glucose levels, both of which can lead to diabetes (American Diabetes Association, 2011b).

### **Inflammation and Asthma**

More than 24.6 million people in the U.S. have been diagnosed with asthma, and the prevalence of asthma has been consistently increasing over the past decade (Centers for Disease Control and Prevention, 2011). Over the past twenty years, research has found persistent airway inflammation to be the main cause of asthma (Dodig, Richter, & Zrinski-Topić, 2011; Nakagome & Nagata, 2011). This inflammation occurs when pro and anti-inflammatory regulatory systems in the body fail to effectively balance inflammatory processes (Dodig et al., 2011). Eosinophils, as well as mast cells and neutrophils, contribute to the onset of asthma by releasing inflammatory mediators in response to allergens (Nakagome & Nagata, 2011). Inflammation in the airway, if untreated, can contribute to asthma. Combined with airway obstruction and airway hyper-responsiveness, chronic inflammation reduces the amount of air that can pass through the lungs. When the airways narrow as a result of these processes, asthmatic-related symptoms occur that include wheezing, shortness of breath, coughing and chest tightness (Asthma, 2011).

### **Inflammation and Rheumatoid Arthritis**

Rheumatoid arthritis affects approximately 2.1 million people in the United States (Kountz & Von Feldt, 2007). This disease is characterized by systemic inflammation, leading to

fatigue, joint pain and general functional decline (Kountz & Von Feldt, 2007; Scott, Wolfe, & Huizinga, 2010). One of the main factors in the development of rheumatoid arthritis (RA) is synovial inflammation in the joints and production of proinflammatory molecules due to an autoimmune response (Harris, 1986). Joint swelling and inflammation of the surrounding tissue is caused by an abundance of proinflammatory molecules, leading to joint stiffness and pain. T-cell activation and the presence of cytokines also contribute to the inflammation associated with RA (Firestein, 1999). Studies have shown that reducing inflammation can help reduce symptoms and improve disease control in rheumatoid arthritis (Blok, Katan, & van der Meer, 1996; Fetterman & Zdanowicz, 2009).

A few of the major inflammatory conditions associated with systemic inflammation have been discussed, but there are several others, such as psoriasis (Blok et al., 1996), inflammatory bowel disease (Uchiyama et al., 2010), sepsis (Barbosa et al., 2010), and renal disease (Bowden, Wilson, Deike, & Gentile, 2009). Collective studies over the past several years show that an increased consumption of n-3 PUFAs, particularly those contained in fish oils, is associated with a decrease in systemic inflammation (Cleland et al., 2006; Fetterman & Zdanowicz, 2009; Wall, Ross, Fitzgerald, & Stanton, 2010). Thus it can be hypothesized that strategies to prevent many of the commonly occurring inflammatory related diseases prevalent in the U.S. may include increasing EPA + DHA in the diet through the consumption of fish or fish oil supplementation. Increasing EPA + DHA consumption may be a cost-effective approach to help prevent many of the inflammatory diseases that are becoming more prevalent worldwide.



**PUFAs—n-6, n-3, EPA and DHA**

Polyunsaturated fatty acids (PUFAs) are important components of cell membranes and also have a role in regulating inflammatory responses through the production of inflammatory mediators called eicosanoids (Wall et al., 2010). PUFAs termed “essential” cannot be synthesized by the human body and must be obtained by diet (Fetterman & Zdanowicz, 2009). There are two main categories of essential PUFAs—n-6 and n-3, which follow similar, but separate metabolic pathways.

Sources of n-6 fatty acids include vegetable oils and animal meats (A. P. Simopoulos, 2000). These n-6 fatty acids are converted by the body into linoleic acid (LA), which is further converted into arachidonic acid (AA). The eicosanoids derived from arachidonic acid include leukotrienes, prostaglandins and thromboxanes that have inflammatory properties (Wall et al., 2010). N-3 fatty acids can be found in canola oil, flaxseed oil, nuts, fish and fish oils (A. P. Simopoulos, 2000). The n-3 PUFAs are converted into alpha-linolenic acids (ALA), which are then converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The eicosanoids derived from EPA and DHA include a series of leukotrienes, prostaglandins and thromboxanes that have anti-inflammatory effects on the body (Wall et al., 2010).

Dietary fish oil rich in omega-3 polyunsaturated fatty acids such as EPA and DHA has been shown to reduce inflammation (Cleland, James & Proudman, 2009). Increasing the consumption of EPA and DHA-rich fish oils smoothes platelet membranes and generates thromboxanes and prostaglandins that have less inflammatory actions than those generated from the n-6 pathway (Cleland et al., 2006). These n-3 PUFAs have beneficial effects on lipid levels, blood pressure, heart function, immunological responses, and other functions of the body (A. P. Simopoulos, 2002a). The Western diet, however, typically contains much lower levels of n-3

PUFAs relative to n-6 PUFAs. Maintaining a relatively balanced n-6:n-3 PUFA ratio may suppress inflammation and the development of autoimmune and inflammatory-related diseases. While our ancestors typically consumed a n-6:n-3 ratio of 1-2:1, today's typical Western diet includes a ratio of about 10:1 – 20:1, indicating that Western diets are deficient in n-3 PUFAs (A. P. Simopoulos, 2000; A. P. Simopoulos, 2008). The best dietary sources of marine-derived n-3 fatty acids are cold-water oily fish, whereas shorter chain n-3 fatty acids, such as linolenic acid, are found in nuts, oils, and seeds. Many have proposed that increasing consumption of these n-3 fatty acids can help promote health (Harris et al., 2010). In order to maintain a beneficial level n-3 PUFAs, particularly EPA and DHA, the American Heart Association recommends eating oily fish twice per week, and that those with coronary heart disease supplement their diets with an additional 1 g/d of EPA plus DHA from oily fish or supplements (Breslow, 2006).

In a study completed by the American Journal of Clinical Nutrition, higher levels of EPA and DHA in the blood were linked to a decreased expression of genes involved in inflammatory and atherogenic pathways (Bouwens et al., 2009). A study conducted by Beluzzi et al. (1996), examined patients with Crohn's disease who received 2.7g/d of EPA and DHA over the course of one year and found that inflammatory markers significantly decreased in the group who took fish oils. In another study that examined the relationship between plasma PUFA levels and inflammatory markers, the results showed that an increase in n-3 PUFA levels was associated with lower levels of proinflammatory markers (including CRP) and higher levels of anti-inflammatory markers (Ferrucci et al., 2006). Other studies have reported that n-3 supplementation with 4-4.5g/d of EPA and DHA lead to substantial reduction in CRP from baseline levels over the course of three years (Cleland, Caughey, James, & Proudman, 2006).

Further evidence that higher n-3 PUFA levels are linked to decreased CRP is provided by Bowden et al. (2009), who found that end-stage renal patients given 2g/d of EPA and DHA fish oil supplementation for six months showed a significant decrease in inflammation from pretest to posttest.

### **C-Reactive Protein**

C-reactive protein (CRP) is a nonspecific marker of inflammation in the blood. This protein is produced in the liver and increases in response to inflammation (Martinez & Gonzalez-Juanatey, 2009). Multiple studies have used CRP as a way to measure systemic inflammation (Biasucci, Biasillo, & Stefanelli, 2010; Bowden et al., 2009; Jiang et al., 2006; Taubes, 2002). CRP has been found to be one of the most effective predictors of heart disease as well as diabetes (Martinez & Gonzalez-Juanatey, 2009; Taubes, 2002). Interestingly, a recent study by Micallef et al. showed that CRP levels were inversely related to total n-3 PUFA and EPA and DHA levels. Therefore, higher CRP levels were associated with significantly lower n-3 PUFA levels and higher levels of inflammation (Micallef, Munro, & Garg, 2009).

Plasma CRP is frequently used as a measure of systemic inflammation, but multiple studies have also shown moderate-to-strong associations between salivary CRP and plasma CRP (Gutiérrez, Martínez-Subiela, Eckersall, & Cerón, 2009; Ouellet-Morin, Danese, Williams, & Arseneault, 2011; Parra, Tecles, Martínez-Subiela, & Cerón, 2005; Pepys & Hirschfield, 2003). The collective evidence suggests that salivary CRP can be used as a non-invasive, valid marker of systemic inflammation.

### **III. Methodology**

#### **Research Design**

This retrospective study used a descriptive, correlation design in which a correlation matrix was used to determine relationships among plasma levels of n-6 and n-3 PUFAs, ratios of n-6:n-3 PUFAs, and salivary CRP levels.

The current study analyzed select data generated from the parent study that were appropriate to explore the relationships of interest. The parent study used a randomized, double-blind, repeated measures design to evaluate the effects of EPA and DHA supplementation on lipid mediator levels in acute wound fluid.

#### **Population and Sample Design**

The participants in the parent study were a convenience sample recruited primarily from a Midwest university area. Participants were healthy individuals ages 18-45 who were able to understand English. Individuals were excluded if they were taking non-steroidal anti-inflammatory drugs, lipid-lowering medications, nutritional supplements, or corticosteroids or were current smokers, pregnant or lactating. A total of 18 participants were enrolled for the study and randomly assigned by computer sort to either the Active group or Placebo group. All participants were blinded as to treatment and provided informed written consent after an explanation of the study.

IRB approval was obtained by investigators of the primary study. Additionally, all research methods conducted were in compliance with the ethical rules for human experimentation stated in the 1975 Declaration of Helsinki.

### **Data Collection Procedures**

Visit 1 took place at the Clinical Research Center (CRC). Demographic data, including age, gender, and race, were collected from all participants. Body measurements included height, weight, sagittal abdominal diameter (SAD) and body mass index (BMI). The primary investigator, who was also blinded to Active and Placebo group assignments, provided written and verbal directions for all participants to take five soft gels and one 81 mg aspirin tablet at bedtime until study completion. Research has shown that low dose aspirin may enhance the actions of the EPA-derived, anti-inflammatory resolvins species (Lichtenstein et al., 2006). The soft gels given to the Active group provided a total of 1.6 g/d of EPA and 1.2 g/d of DHA. Previous studies using similar doses of n-3 fatty acid supplementation have resulted in lower levels of proinflammatory markers (Belluzi et al., 1996; Cleland, Caughey, James & Proudman, 2006; Bowden et al., 2009). The Placebo group received soft gels providing a total daily intake of 2.4 ml of mineral oil, which is well below the therapeutic dose recommended for constipation and remains mostly unabsorbed in the feces. All soft gels had the same appearance and packaging in containers provided by J.R. Carlson Laboratories, Inc. (Arlington Heights, IL). A specific date to begin the soft gels and aspirin was given to each participant. All participants were instructed to exclude fish, seafood, kelp and flaxseeds from their diets until study completion because these foods contain omega-3 fatty acids and could interfere with study results. Blood was collected after an 8-hour fast for plasma fatty acid analysis. Electronic Food Frequency Questionnaires (FFQ) (VioFFQ, Viocare, Inc., Princeton, NJ) were completed by all participants. The FFQs were used to estimate micro and macro nutrient intake during the three months prior to completing the questionnaire. This data allowed a baseline comparison of nutrient intake, including PUFAs, between the Active and Placebo groups.

Visit 2 occurred four weeks after participants began taking the soft gels. Each participant was admitted to the CRC for completion of the blistering protocol. Blood samples were collected for plasma fatty acid analyses, saliva was collected to evaluate levels of cortisol during the blistering procedure, and body measurements were recorded. The salivary samples in this study were taken from the parent study to determine CRP levels.

### **Data Collection Instruments**

**Plasma fatty acid assays.** Plasma fatty acids were quantified by gas chromatography/mass spectrometry (GC/MS) at the laboratory in the College of Education and Human Ecology, OSU (Columbus, OH). Blood samples (1.0mL) were collected in EDTA vacutainers, centrifuged at 720 g for 30 minutes at room temperature to isolate the plasma fraction and stored at -80°C prior to analysis. Total lipids were extracted from plasma samples with 2:1 (v/v) chloroform:methanol and 0.24ml 0.88% KCL. Fatty acid methyl esters were prepared using tetramethyguandine at 100°C. Analysis of fatty acid methyl esters was completed by gas chromatography using a 30-m Omegawax <sup>TM</sup> 320 fused silica capillary column (Supelco, Bellefonte, PA). Oven temperature started at 175°C and increased at a rate of 3°C/min until reaching 220°C. Flow rate of the carrier gas helium was 30mL/min. Retention times of samples were compared to standards for fatty acid methyl esters (Matreya, LLC, Pleasant Gap, PA, Supelco, Bellefonte, PA, and Nu-Check Prep Inc., Elysian, MN). Fatty acids are reported as prefect area of total fatty acids identified in plasma samples.

**C-reactive protein assays.** Using the salivary samples from the parent study, CRP was measured at 12 and 24 hours post wounding to determine mean levels at Visit 2. Studies comparing salivary CRP to CRP in serum, a commonly used biomarker of systemic

inflammation, have shown associations that suggest salivary CRP is a non-invasive, valid marker of systemic inflammation (Gutiérrez et al., 2009; Ouellet-Morin et al., 2011). All samples were assayed for salivary CRP in duplicate using a highly sensitive enzyme immunoassay (Salimetrics, State College, PA). The test used 50 µl of a 10x dilution of saliva per determination, has a lower limit sensitivity of 10pg/mL, a standard curve range from 93.75 to 3000pg/mL, an average intra-assay coefficient of variation of 3.9% and an average inter-assay coefficient of variation of 7.5%. Method accuracy determined by spike and recovery averaged 98.9% and linearity determined by serial dilution averaged 96.2%.

### **Data analysis**

Statistical analyses were completed using the SPSS statistical package for WINDOWS, version 19 (SPSS Inc., Chicago, IL). T-tests were used for between group comparisons of sociodemographic data, body measurements, and nutritional data generated by the FFQs.

Statistical analysis of the experimental data was completed in three steps to evaluate associations among the variables of interest. The first step was to quantify the strength of relationship between various PUFA measures and salivary levels of CRP. Measured values of CRP and measured values of several PUFAs exhibited a right-skewed distribution, so a non-parametric measure of association was used (Kendal's tau-β). The second step was to quantify the strength of relationship between the treatment group and PUFA measures. Since several of the PUFA measures were right skewed, a nonparametric Mann-Whitney test was used to test for differences between the Active and Placebo Groups at baseline and 4 weeks. To determine whether PUFA levels changed significantly between the two time points (baseline and 4 weeks) within each group, separate Wilcoxon tests were performed for each of the PUFA measures. The

final step was to quantify the strength of relationship between treatment group and CRP. Since the dependent variable was heavily right-skewed, a nonparametric Mann-Whitney test was used. Significance levels were set a priori at  $p < 0.05$ .

## **IV. Research Results**

### **Profile of Sample/Population**

Anthropometric measures and demographic characteristics describing participants in the group as a whole and by Active and Placebo groups are displayed in **Table 1** with similar data for both groups. There were no significant differences in male-to-female ratio, mean age, BMI, or SAD between the Active and Placebo groups.

Evaluation of the FFQ data revealed that the mean daily dietary intake of the n-6 PUFA linoleic acid and AA and the n-3 PUFA linolenic acid, EPA and DHA from foods consumed in the 3 months prior to the study did not differ significantly across groups at baseline (**Table 2**). Furthermore, there were no significant differences in mean daily intakes of iron, zinc, copper and magnesium between the 2 groups. These latter elements can affect fatty acid metabolism because the desaturases are metalloenzymes (Arab, 2003).

There were no significant correlations detected between PUFA measures assessed by the FFQs completed at baseline with the corresponding plasma PUFAs measured at baseline, however, plasma concentrations of AA, linolenic acid, EPA and DHA were positively correlated with the dietary intake of the corresponding PUFA.

### **C-Reactive Protein**

To test Hypothesis 1, we performed a statistical analysis of salivary CRP that quantified the strength of relationship between various PUFA measures and CRP concentrations detected in



the saliva. We observed a significant negative correlation between CRP and total n-3 PUFAs ( $\text{tau-}\beta = -0.373$ ,  $p = 0.031$ ) (**Figure 2**).

A marginally significant positive correlation was detected between CRP and the ratio of n-6 to n-3 ( $\text{tau-}\beta = 0.320$ ,  $p = 0.063$ ) (**Figure 3**). Total AA was not significantly correlated with CRP ( $\text{tau-}\beta = 0.03$ ), nor were EPA and DHA (EPA:  $\text{tau-}\beta = -0.19$ ; DHA:  $\text{tau-}\beta = -0.27$ ). BMI had a non-significant positive correlation with CRP (BMI:  $\text{tau-}\beta = 0.11$ ), similar to previous studies (Bermudez, Rifai, Buring, Manson, & Ridker, 2002; Yamamoto, Okazaki, & Ohmori, 2011).

The mean CRP concentration (0.001 mg/L) for the Active Group was lower than that of the Placebo Group (0.039 mg/L), but the difference was not statistically significant ( $p = 0.15$ ). CRP values ranged from 0.000098 mg/L (98 pg/mL) to 0.118796 mg/L (118796 pg/mL) for the combined group ( $M = 0.009$  mg/L [9000 pg/mL],  $SD = 0.03$  [30,000 pg/mL]).

### **Plasma Fatty Acids**

To test Hypothesis 2, we analyzed the effects of the study dose on plasma PUFAs by first evaluating the changes in PUFA concentrations from baseline to Week 4 within each group using separate Wilcoxon tests. As expected, in the Active Group, time was found to be significantly related to all PUFAs measured except for AA and total n-6 PUFAs. For EPA, DHA, and total n-3 PUFAs, the average subject was found to have a significant increase with treatment ( $p = 0.008$  in all cases) (**Table 3**).

For the ratio of n-6 to n-3 and the ratio of AA to EPA, the average subject was found to have a significant decrease with treatment ( $p = 0.008$  in all cases). The data suggest that rising EPA concentrations were the primary contributing factors to the change in the AA:EPA ratios. In the Placebo Group, time was found to be significantly related to DHA, total n-3 and

total n-6 PUFAs. For all three of these variables, the average subject was found to have a significant decrease over the course of treatment with a placebo ( $p = 0.008$ ,  $p = 0.011$ , and  $p = 0.038$  for DHA, total n-3 and total n-6, respectively). Collectively, the data indicated that the EPA+DHA supplements were taken appropriately by participants in the Active Group and that the dose and duration of supplementation were adequate to significantly raise plasma concentrations of EPA, DHA and total n-3 PUFAs and significantly reduce both the total n-6:n-3 and AA:EPA ratios from baseline values.

The strength of relationship between treatment group and PUFA measures was then evaluated. Group assignment was found to be significantly related to all PUFA measures except AA and total n-6 PUFAs at Week 4 (**Table 3**). For the ratio of n-6 to n-3 and the ratio of AA to EPA, the Active Group had significantly lower levels than the Placebo Group ( $p < 0.001$  in both cases). Similarly, for EPA, DHA, and total n-3 PUFAs, the Active Group had a significantly higher level than the Placebo Group after four weeks of EPA and DHA supplementation ( $p < 0.001$  in all cases).

The strength of relationship between treatment group and CRP, using a nonparametric Mann-Whitney test was not significant (0.161). As a secondary check on this relationship, CRP was transformed to the log scale, resulting in an approximately normal distribution of log-CRP values. The transformed data was examined using a two-sample t-test and no significant relationship was found ( $p = 0.179$ ).

## **V. Summary, Conclusions and Recommendations**

### **Summary of Findings**

The main purpose of our study was to evaluate the effects of n-3 EPA and DHA oral supplementation on plasma fatty acid levels and systemic inflammation. Our data indicates

significant changes of PUFA levels after 4 weeks of supplementation with 1.6 g/d of EPA and 1.2 g/d DHA. We found that the active group had significantly higher mean levels of EPA and DHA and a lower AA/EPA ratio after the course of 4 weeks. EPA increased from an average of 0.38 to 3.27, and DHA increased from 1.55 to 4.02. The AA/EPA ratio decreased from 25.6 and 2.65 between the baseline and second visit. This shows that the fish oil supplementation significantly affects the levels of plasma fatty acids; oral supplementation with EPA+DHA is associated with higher plasma concentrations of n-3 PUFAs and lower n-6:n-3 ratios. Additionally, CRP levels were lower for the active group at 4 weeks when compared to the placebo group. The data indicate that higher plasma concentrations of total n-3 PUFAs are significantly associated with lower CRP concentrations and that a positive correlation approaching significance exists between CRP and n-6:n-3 ratios. Our findings are consistent with other studies reporting that n-3 PUFAs have anti-inflammatory actions (Calder, 2010; Ferrucci et al., 2006; Micallef et al., 2009).

## **Conclusions**

In summary, we report that total plasma n-3 PUFAs are inversely correlated with CRP in healthy younger adults. We also report that n-3 EPA+DHA oral supplementation for four weeks is positively correlated with higher plasma concentrations of total n-3 PUFAs, EPA and DHA and lower n-6:n-3 ratios. Our findings that higher total n-3 PUFA concentrations and lower n-6:n-3 ratios in plasma are correlated with decreasing CRP concentrations in a sample of younger adults are aligned with a recent study by Ferrucci et al. (2006), who evaluated the relationships of plasma PUFA concentrations to circulating inflammatory markers in 1,123 persons. However, their population sample included much older adults (aged 20-92 years). They reported that PUFAs, and especially total n-3 PUFAs, were independently associated with

lower levels of proinflammatory markers (CRP, IL-6, IL-1ra, and TNF- $\alpha$ ) and higher levels of anti-inflammatory markers (soluble IL-6r, IL-10, TGF $\beta$ ) independent of confounders. The present study findings are also in agreement with a study conducted by Micallef et al. (2009), which demonstrated an inverse relationship between the intake of n-3 PUFAs and biomarkers of inflammation in human subjects ages 35-59.

### **Limitations**

A limitation of this study is the small number of participants (n=18) who were all from the same geographical area. Therefore, the results of our study may not generalize to other geographic locations. Additionally, only one specific dose of EPA/DHA was used in the study. Thus, it is not known if varied doses of n-3 PUFAs would produce the same effects on plasma PUFA and CRP levels as noted in this study.

### **Implications of Study**

Findings from this study support previous assessments that there are health benefits to increasing n-3 EPA+DHA consumption either through foods or supplements. The most beneficial n-6:n-3 ratio may differ across clinical populations, but a more balanced ratio is associated with normal development and homeostasis in terms of eicosanoid metabolism and cytokine production. It is believed that a smaller ratio of n-6:n-3 PUFAs results in less inflammation and the possible improvement or prevention of many diseases that have an inflammatory component (A. P. Simopoulos, 2008). Additional studies are needed to explore this promising strategy for helping prevent many of the major chronic diseases associated with excessive, protracted inflammation, such as CVD, in the younger adult population.

**Recommendations**

The U.S. Federal Drug Administration (FDA) has evaluated the safety of EPA and DHA and concluded that a daily intake of EPA+DHA of up to 3.0 g/d is acceptable for the general public to achieve anti-inflammatory effects (U.S. Food and Drug Administration, 2009). However, the optimal dose of n-3 PUFAs for primary risk reduction in the younger adult population is still unclear. Future studies investigating the associations between diets supplemented with PUFAs, plasma PUFA concentrations and inflammatory markers in this population segment should consider testing more than one dose of n-3 EPA+DHA in a larger sample of younger adults and evaluating inflammatory markers pre and post supplementation.

## References

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Table 1. Baseline Characteristics of the Study Participants by Group

	Active <sup>a,b,c</sup> (n = 9)	Placebo <sup>a,b,c</sup> (n = 9)
Age, mean years	24 (5.4)	28 (8.4)
Male	2	7
White	8	7
African American	0	1
Asian	1	0
Other	0	1
Anthropometric measures		
Height, centimeters	168.9 (6.2)	176.4 (10.2)
Weight, kilograms		
Baseline	73.3 (20.3)	79.4 (12.6)
4 weeks	73.5 (21.1)	79.5 (13.2)
BMI, kilograms/meter <sup>2</sup>		
Baseline	25.8 (7.7)	25.6 (3.8)
4 weeks	25.8 (7.9)	25.6 (3.8)
SAD, centimeters		
Baseline	19.3 (4.3)	19.7 (1.7)
4 weeks	19.3 (3.8)	20.0 (2.2)

<sup>a</sup> No significant differences between groups<sup>b</sup> Analyzed with t-test<sup>c</sup> Mean (SD) (all such values)

BMI = body mass index; SAD = sagittal abdominal diameter

Table 2. Dietary Intake of n-6 and n-3 PUFA per Food Frequency Questionnaires (FFQ) by Group at Baseline

	Active <sup>a,b,c</sup> (Baseline) (n=9)	Placebo <sup>a,b,c</sup> (Baseline) (n=9)
<b>Dietary Intake</b>		
n-6 PUFA (g/d)		
Linoleic acid (18:2n-6)	12.37 (5.67)	16.55 (8.90)
AA (20:4n-6)	0.12 (0.11)	0.15 (0.09)
n-3 PUFA (g/d)		
Linolenic acid (18:3n-3)	1.30 (0.61)	1.60 (0.98)
EPA (20:5n-3)	0.03 (0.03)	0.02 (0.01)
DHA (22:6n-3)	0.07 (0.08)	0.05 (0.03)

<sup>a</sup> No significant differences between groups<sup>b</sup> Analyzed with Mann-Whitney test<sup>c</sup> Mean (SD)

PUFA = polyunsaturated fatty acids; AA = arachidonic acid; EPA = eicosapentaenoic acid;  
DHA = docosahexaenoic acid.

Table 3. Plasma Fatty Acid Measures at Baseline and After 4-week Supplement Intervention of EPA+DHA or Placebo of Mineral Oil

Measures of Plasma Fatty Acid for Active and Placebo Group - Mean (SD)						
Fatty Acids ( % area of total fatty acids)	Baseline AC (n=9)	Baseline PL (n=9)	Week 4 AC (n=9)	Week 4 PL (n=9)	Change AC (n=9)	Change PL (n=9)
Ratios						
n-6/n-3	10.99 (2.04)	9.40 (1.39)	4.12 (0.67) <sup>a,d</sup>	10.77 (1.70) <sup>d</sup>	-6.88 (2.21) <sup>a,d</sup>	1.37 (1.99) <sup>d</sup>
AA/EPA	25.60 (11.84)	20.79 (7.79)	2.65 (0.65) <sup>a,d</sup>	21.89 (4.84) <sup>d</sup>	-22.95 (11.93) <sup>a,d</sup>	1.10 (9.48) <sup>d</sup>
Total						
n-6	41.69 (6.74)	43.47 (3.67)	39.09 (4.44)	40.41 (2.86) <sup>c</sup>	-2.60 (6.67)	-3.06 (4.19) <sup>c</sup>
n-3	3.83 (0.45)	4.73 (0.85)	9.70 (1.82) <sup>a,d</sup>	3.83 (0.60) <sup>b,d</sup>	5.87 (2.06) <sup>a,d</sup>	-0.90 (0.81) <sup>b,d</sup>
Fatty Acids						
EPA	0.38 (0.15)	0.57 (0.22)	3.27 (0.91) <sup>a,d</sup>	0.43 (0.18) <sup>d</sup>	2.89 (0.98) <sup>a,d</sup>	-0.14 (0.20) <sup>d</sup>
DHA	1.55 (0.42)	1.84 (0.55)	4.02 (0.73) <sup>a,d</sup>	1.29 (0.43) <sup>a,d</sup>	2.47 (0.93) <sup>a,d</sup>	-0.55 (0.31) <sup>a,d</sup>
AA	8.49 (2.59)	10.74 (2.64)	8.25 (1.48)	9.02 (2.53)	-0.25 (2.24)	-1.72 (2.18)

AC = Active; PL = Placebo; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; AA = arachidonic acid

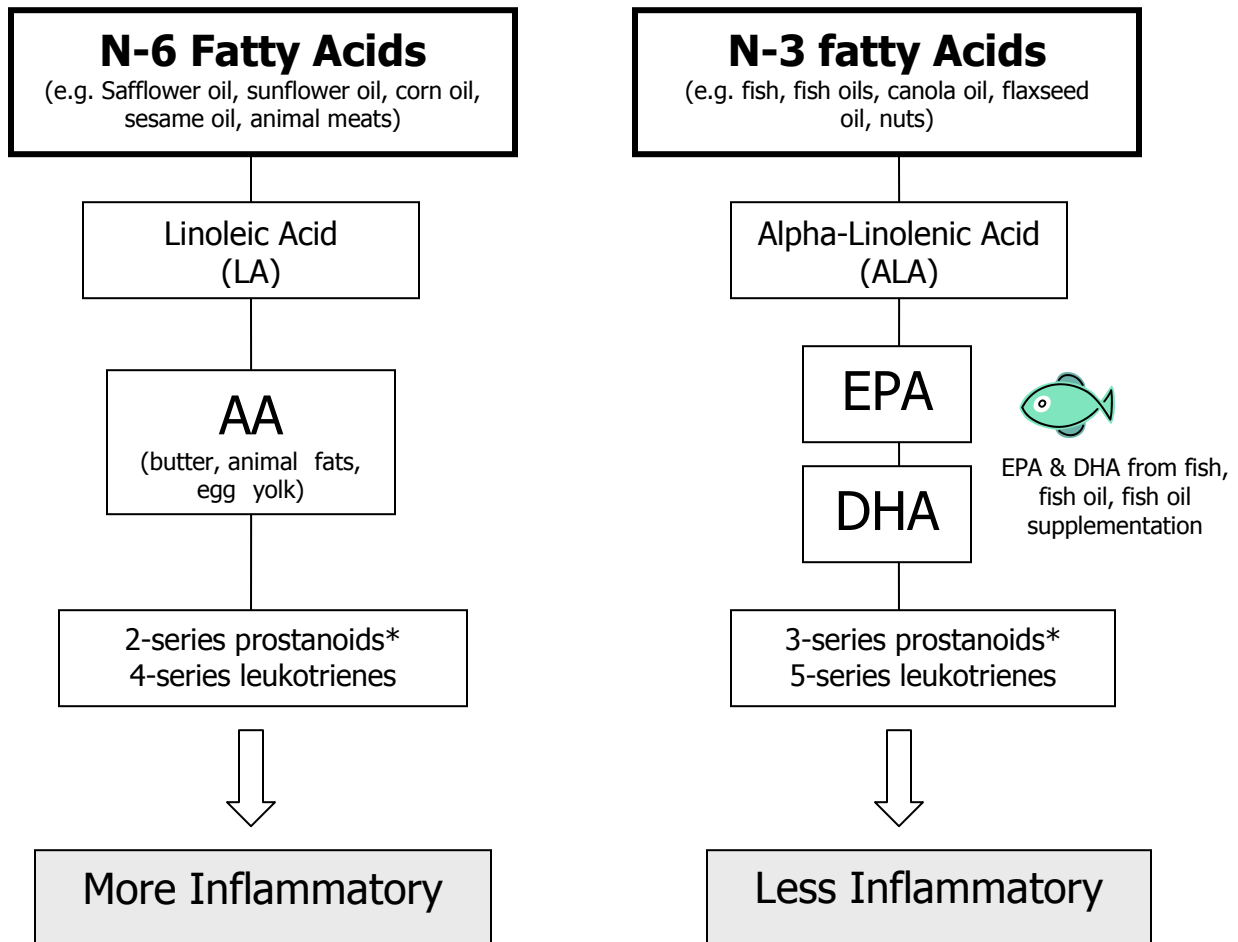
a = significantly different from baseline—within group by Wilcoxon test (p = 0.008)

b = significantly different from baseline—within group by Wilcoxon test (p = 0.011)

c = significantly different from baseline—within group by Wilcoxon test (p = 0.038)

d = significantly different between groups @ wk. 4 by Mann-Whitney test (p < 0.001)

Figure 1. N-6 and n-3 Polyunsaturated fatty acid metabolic pathways



AA= Arachidonic acid; DHA= Docosahexaenoic acid; EPA= Eicosapentaenoic acid

\*Prostanoids are a subclass of eicosanoids and include prostaglandins, thromboxanes, and prostacyclins



Figure 2. Relationship between total n-3 PUFA levels in plasma and CRP levels

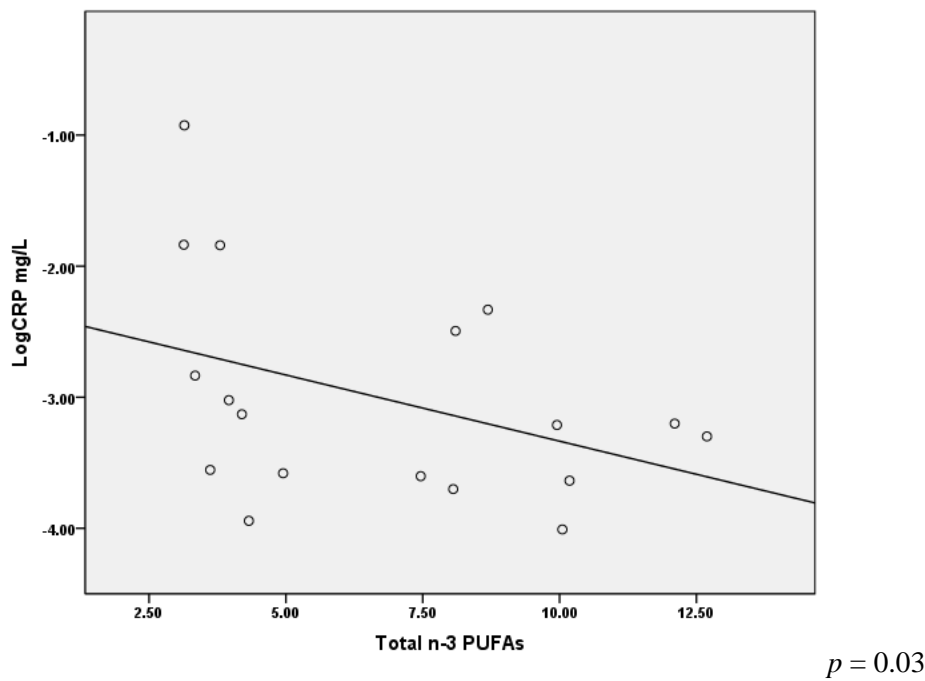


Figure 3. Relationship between n-6:n-3 ratios in plasma and CRP levels

